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# From Field to Sequencer: Methodological Impacts of Sampling, Ethanol Storage, and DNA Extraction on Equine Fecal Microbiota (16S Amplicon Sequencing)

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# From Field to Sequencer: Methodological Impacts of Sampling, Ethanol Storage, and DNA Extraction on Equine Fecal Microbiota (16S Amplicon Sequencing)

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## Structured abstract

**Background**
Profiling of the equine fecal microbiota with 16S amplicon sequencing is complicated by field--dependent sample preservation, identification of inhibitory compounds in feces, and determination of the degree of technical variation during DNA extraction and sequencing. The relative impacts of these methodological steps, however, remain poorly quantified in horses. This study evaluated how extraction kits, sequencing platforms, and long-term ethanol storage affect equine fecal microbiota characterization.

**Methods**
Fecal samples (n = 12; 4 horses × 3 biological replicates) were extracted via two commercial kits (QIAamp Fast DNA Stool Mini and DNeasy PowerFecal Pro), sequenced via Illumina NovaSeq and Ion S5, and processed via cutadapt, DADA2, VSEARCH, and SILVA v138.2. Storage effects were assessed on ethanol-preserved Fast Stool extracts stored at 4 °C or room temperature for up to 13 months. Community variation was evaluated via Bray–Curtis dissimilarity, PCoA, PERMANOVA with appropriate permutation constraints, and betadisper.

**Results**
Horse identity explained the largest share of microbiota variation across all analyses ($R^2 \approx 0.26$–$0.34$, $p \leq 0.001$). The sequencing platform contributed additional variation ($R^2 = 0.099$, $p \leq 0.001$), and Illumina yielded substantially greater read depth and OTU richness than Ion S5. The extraction kit introduced significant but heterogeneous compositional shifts ($R^2 \approx 0.07$–$0.08$), including kit-specific OTU sets and multiple family-level differences. Long-term ethanol storage produced statistically detectable but modest shifts ($R^2 \approx 0.08$), with no differences in dispersion across storage conditions or extraction dates. Across all analyses, inter-individual variation exceeded methodological effects.

**Conclusions**
In equine fecal 16S amplicon sequencing, biological individuality dominates over methodological variation. Nevertheless, the sequencing platform, extraction kit, and long-term ethanol storage each introduce measurable compositional differences. These findings underscore the importance of standardized extraction protocols and transparent reporting of preservation and sequencing parameters to improve reproducibility and comparability in equine microbiota studies.

# Introduction

Historically, microbiome research has focused predominantly on human subjects. In recent years, however, the field has expanded to encompass a wide range of animal species, often motivated by questions in ecology, evolution, and conservation biology [1–5]. The gut microbiome plays a pivotal role in mediating the physiological effects of diet and is a critical component of overall animal health and survival [6–9]. While the term microbiome is defined as "a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties," the term microbiota refers to the living members forming this community [10]. The composition of the microbiota differs markedly along the gastrointestinal tract [11, 12] and is shaped by factors such as diet, age, health, and stress [13, 14]. Advances in sequencing-based methods, including 16S rRNA gene amplicon sequencing, have enabled detailed characterization of these communities across host species. Conventional culture-based approaches have largely been replaced by sequencing-based methods that enable comprehensive characterization of the gut microbiota [15]. Among these methods, amplicon-based sequencing of the 16S rRNA gene remains the most widely applied method for profiling microbial communities [16–18].

Fecal samples are widely used to study the gut microbiota because they can be collected non-invasively and can be used to assess diet, health, disease, and environmental influences [19–21]. Although the extent to which fecal samples represent the entire gastrointestinal community has been questioned [22, 23], they remain a practical and essential tool for studies in large animals where more invasive sampling is not feasible. The reliability of these studies, however, depends heavily on methodological choices, such as sampling strategy, preservation, DNA extraction, and sequencing platform, each of which introduces potential sources of bias [24–28].

Preservation is a particular challenge in field studies where immediate freezing at −80 °C is impractical. Ethanol is one of the most commonly used preservatives and has been shown to stabilize DNA effectively [27, 29]. In human studies, the preservation of DNA from fecal samples with 95% ethanol has proven effective, even for long-term storage [27]. However, because the fecal composition and microbiota differ widely across species [30–32], ethanol-based preservation requires species-specific validation. The impact of long-term ethanol storage at different temperatures remains poorly characterized in non-human hosts [11]. The drawbacks of using ethanol include the risk of PCR inhibition if residual ethanol is not removed [33]. Although short-term storage at 4 °C appears relatively stable for up to 96 h [34], the long-term effects in equids remain unknown.

Numerous studies have demonstrated that the choice of DNA extraction method significantly influences the observed microbial community composition and diversity, underscoring the need for careful method selection and standardization, including in stool samples [25, 35–41]. Different DNA extraction kits may vary in their ability to effectively lyse the robust cell walls of gram-positive bacteria, which possess a thicker and more rigid peptidoglycan layer. Inefficient lysis can lead to underrepresentation of these taxa in downstream microbiome analyses, potentially biasing the results [42, 43]. Furthermore, the efficiency of DNA extraction methods has been reported to vary

across species [40], and fecal inhibitors such as humic substances, bile pigments, and polysaccharides may impact both DNA yield and downstream analyses [23, 24].

High-throughput sequencing has become a central tool for microbial community profiling, with 16S rRNA gene amplicon sequencing widely applied across environments, species, and experimental systems. Illumina NovaSeq is one of the most commonly used platforms because of its low error rates, high sequencing accuracy, and ability to generate deep coverage suitable for resolving bacterial community structure [44]. Alternative technologies such as Ion Torrent remain viable options for targeted applications, particularly in laboratories where these platforms are more readily available or offer advantages in cost, turnaround time, or workflow simplicity [45]. However, differences in sequencing chemistry, read length, and platform-specific error profiles can introduce systematic biases in estimates of microbial diversity and taxonomic composition [45]. Sequencing depth is a particularly critical factor, as it strongly influences the detection of low-abundance taxa, which may be represented by only a small number of reads or operational taxonomic units (OTUs) [46]. Understanding how platform choice and sequencing depth shape amplicon-based microbiota profiles is essential for designing metabarcoding studies and evaluating methodological comparability across workflows.

In equids, the hindgut microbiota is critical for cellulose fermentation and overall health [47, 48]. In domesticated Equidae, various gastrointestinal diseases, including colitis [49] and laminitis [50], have been linked to imbalances in the gut microbiota (dysbiosis) [51, 52]. Despite the clear link between gut health and disease, our understanding of the factors influencing the equine gut microbiota remains limited [51, 53–55]. Horses are well suited for fecal-based microbiota studies: they produce abundant feces, which facilitate replicate sampling, and the physical properties of their dung provide some protection against DNA degradation [56]. It has been shown that horse fecal samples remain stable for up to six hours after defecation [57, 58]. Several studies have also shown that the equine hindgut microbiota is highly individualized and relatively stable over time, with strong host-specific signatures that persist despite dietary or environmental variation [8, 51–53]. The fiber-rich composition of equid feces and their high inhibitor content pose methodological challenges that differ from those of the protein-rich feces of carnivores and omnivores, underscoring the need for equid-specific extraction protocols and methodological evaluations tailored to equids [59].

Despite the importance of methodological consistency, the combined effects of extraction kits, sequencing platforms, and long-term ethanol storage have not been systematically evaluated in horses. In this study, we addressed this gap by analyzing fecal samples from four horses via two commercial extraction kits, two sequencing platforms, and long-term ethanol storage over 13 months, with biological and extraction replicates processed under standardized protocols. This design allows us to quantify the relative contributions of the host, extraction chemistry, platform differences, and storage conditions to equine fecal microbiota composition.

# Materials and methods

## Study design and sampling

Fecal samples were collected from four privately owned male horses (aged 2, 4, 27, and 32 years). The horses were housed in a private stable and collected in Bø in Telemark, Norway, July 2022. The horses were maintained on a consistent diet of hay and commercial horse feed, with no history of gastrointestinal issues or antibiotic treatment for at least three months prior to sampling. Each sample was assigned a unique identifier based on the horse (H1–H4) and biological replicate (BR1–

BR3). Fresh fecal samples (< 2 h post-defecation) were collected via sterile gloves, which were changed between samples to prevent cross-contamination. Each sample was placed into a sterile 50 mL tube and submerged in > 98% denatured ethanol. From each horse, three biological replicates were collected from separate fecal boli within the same defecation event, yielding a total of 12 samples (4 horses × 3 replicates). The samples were transported to the laboratory within 4 h and processed for DNA extraction the same day.

## DNA Extraction Procedures

Fecal samples were processed for DNA extraction via two commercially available kits: the DNeasy PowerFecal Pro Kit (Qiagen; hereafter PowerFecal Pro) and the QIAamp Fast DNA Stool Mini Kit (Qiagen; hereafter Fast Stool). A standardized pre-extraction PBS wash and bead-beating step was applied to all the samples. For all extractions, 1 mL of homogenized fecal slurry was used per kit. For each sample, three extraction replicates were performed. Negative extraction controls (ethanol blanks) were included every 12 samples per kit, and positive controls used the ZymoBIOMICS Microbial Community Standard. One Fast-Stool extract (H2, sample ID 316) was lost during processing. The detailed protocols and deviations from the manufacturer's instructions are provided in the Supplementary Methods.

### Storage experiment

To assess long-term ethanol storage, the fecal samples were divided in half and stored at either 4 °C or room temperature (~21 °C) for up to 13 months. Re-extractions were performed at three time points: ~10 weeks, 16 weeks, and 1 year after initial extraction. The samples were processed via the QIAamp Fast DNA Stool Mini Kit and sequenced on the Illumina sequencing platform.

## Amplicon sequencing

The bacterial V4 region (515F/806R; Apprill/Parada- modified [16, 60, 61]) was amplified and sequenced on both the Ion Torrent (Ion S5, 530 chip) and Illumina (NovaSeq 6000, 2×250 bp) platforms. Library preparation, normalization, and instrument--specific procedures followed vendor recommendations with minor adjustments; full PCR recipes, thermocycling conditions, normalization and quantification steps, chip/flowcell details, and quality control are provided in the Supplementary Methods.

## Bioinformatics

The reads were adapter- and primer- trimmed and demultiplexed via Cutadapt [62]. The Illumina and Ion Torrent datasets were then processed separately in DADA2 [63] with platform-specific filtering parameters (Illumina paired-end settings; Ion Torrent settings adjusted for homopolymer-associated indel errors). After ASV inference, identical sequences were merged across platforms via VSEARCH, chimeras were screened, and taxonomy was assigned against the SILVA v138.2 reference database. The detailed parameter settings (filterAndTrim, learnErrors, dada, mergePairs, removeBimeraDenovo), VSEARCH commands, and software versions are provided in the Supplementary Methods.

## Statistics

All analyses were performed in R via phyloseq [64], vegan[65], ggplot2 [66], and associated packages. Beta diversity was calculated via Bray–Curtis distances on relative abundances, ordination was performed via PCoA, and effects were tested via PERMANOVA (adonis2) with appropriate permutation constraints (strata) for paired or nested designs. The homogeneity of multivariate dispersion was assessed via betadisper. Platform differences were further evaluated via paired

Wilcoxon tests with Benjamini–Hochberg false discovery rate (FDR) correction. Differential abundance was assessed via DESeq2 [67] with the design formula ~ horse_name + extraction_kit. As a sensitivity analysis, all samples were rarefied to 10,000 reads before re-running ordination and PERMANOVA. The full model specifications, permutation numbers, and package versions are listed in the Supplementary Methods.

### Interest list of bacteria

A set of 19 bacterial families of documented relevance to the equine hindgut microbiome was defined a priori for evaluating extraction-kit effects on the basis of commonly cited foundational literature in the field [8, 36, 42, 47, 48, 58]. The full list is provided in Supplementary Table S1.

## Results

### Overall effects on community composition

Overall, host identity was the dominant driver of community composition, whereas methodological factors such as sequencing platform, extraction kit, and storage conditions had relatively small but significant effects (see Figure 1). To account for dataset structure, two PERMANOVA models were used: Model 1 (n=159) tested sequencing platform effects via samples sequenced on both the Illumina and Ion Torrent platforms, whereas Model 2 (n=121) tested storage effects using Illumina-only storage samples originating from different libraries within the same sequencing run were used. PERMANOVA revealed that horse identity was the strongest determinant of fecal microbiota composition (Model 1: $R^2 = 0.258$, $p \leq 0.001$; Model 2: $R^2 = 0.339$, $p \leq 0.001$). Among the methodological factors, the sequencing platform explained the largest share of variance in the matched Library 1 design ($R^2 = 0.099$, $p \leq 0.001$), followed by the extraction kit (Model 1: $R^2 = 0.080$, $p \leq 0.001$; Model 2: $R^2 = 0.071$, $p \leq 0.001$) and storage conditions (Model 2: $R^2 = 0.076$, $p \leq 0.001$). The biological replicate factor contributed the least, but it remained significant in Model 1 ($R^2 = 0.024$, $p \leq 0.01$). The homogeneity of multivariate dispersion was assessed with PERMDISP. Dispersion differed between sequencing platforms (Model 1: $p = 0.001$), whereas storage levels were not significantly different (Model 2: $p = 0.291$).

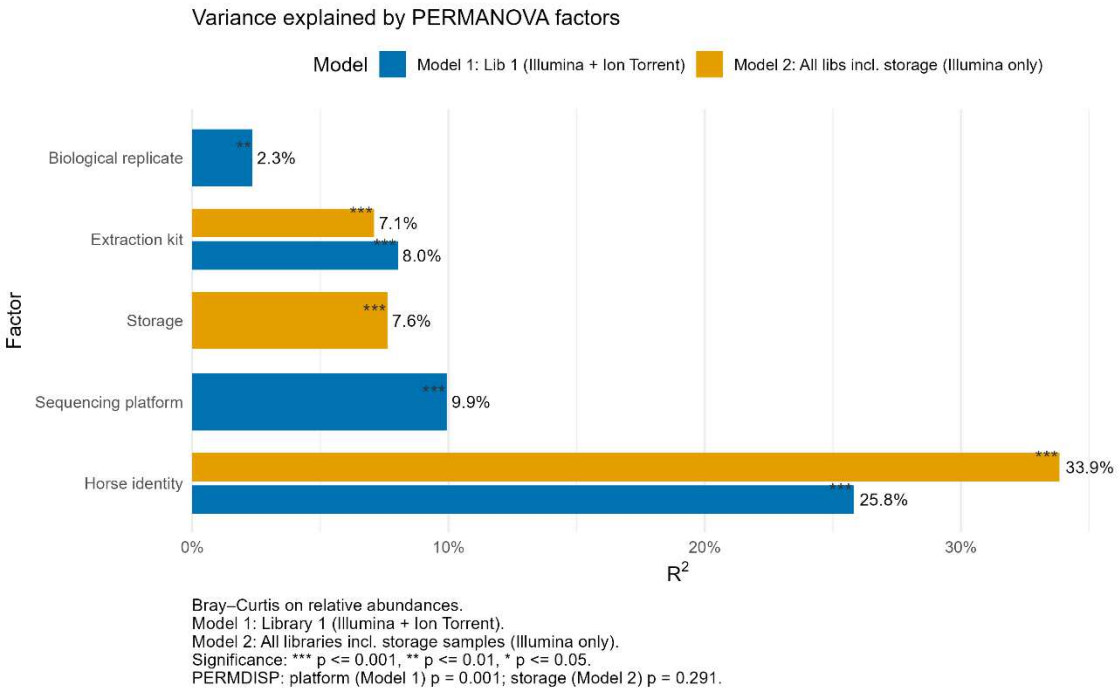



**Figure 1. Variance explained by PERMANOVA factors.** Barplot showing marginal $R^2$ values for Model 1 (Illumina + Ion Torrent; permutations stratified by horse) and Model 2 (Illumina only; permutations stratified by library). Significance levels: ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05. PERMDISP results indicated dispersion differences for platform but not for storage.

## Effect of sequencing platforms on microbiota profiles

The sequencing depth and observed OTU richness differed between the platforms. Compared with Ion Torrent, Illumina yielded higher read counts (median 358,305; IQR ≈ 713,504) (median 10,474; IQR ≈ 18,770), resulting in higher OTU richness (Illumina median 1,638 vs. Ion Torrent median 64; Supplementary Figure S1). Platform separation was evident in the Bray–Curtis PCoA ordinations and persisted after rarefaction to 10,000 reads, while the samples continued to cluster by horse within each platform. The platform groups also differed in multivariate dispersion (PERMDISP: $F = 148.46$, $p = 0.001$) (see Supplementary Figure S2). After rarefaction, dispersion differences persisted (PERMDISP: F = 61.99, p = 0.001). No phyla were uniquely detected by Ion Torrent, whereas three phyla were exclusive to Illumina at ≥0.1% relative abundance (Acidobacteriota, Bdellovibrionota, and Fusobacteriota). Major phylum-level differences are shown in Supplementary Figure S3, including pronounced shifts in Bacillota, Bacteroidota, Actinomycetota and Pseudomonadota, which were significantly more abundant in the Illumina libraries (Wilcoxon p values < $10^{-12}$ for several phyla).

## DNA extraction kit

DNA extraction kits differ in community composition, with kit-- and platform--specific OTU sets visualized in the UpSet plot (Figure 2). Within-horse effects were evident in the Bray–Curtis PCoA faceted by horses, where samples were separated by an extraction kit (Supplementary Figure S4). Alpha diversity showed no systematic kit effect for most horses, with differences largely driven by H2 (Supplementary Figure S5A). The DNA concentration followed a similar horse--specific pattern (Supplementary Figure S5B). PERMANOVA with horses as strata indicated a significant kit effect ($R^2 = 0.153$, $p = 1\times10^{-4}$), and betadisper ANOVA ($F = 21.17$, $p = 1.76\times10^{-5}$) revealed heterogeneous

dispersion between kits. The family-level differential abundance between kits is presented in Supplementary Figure S6.

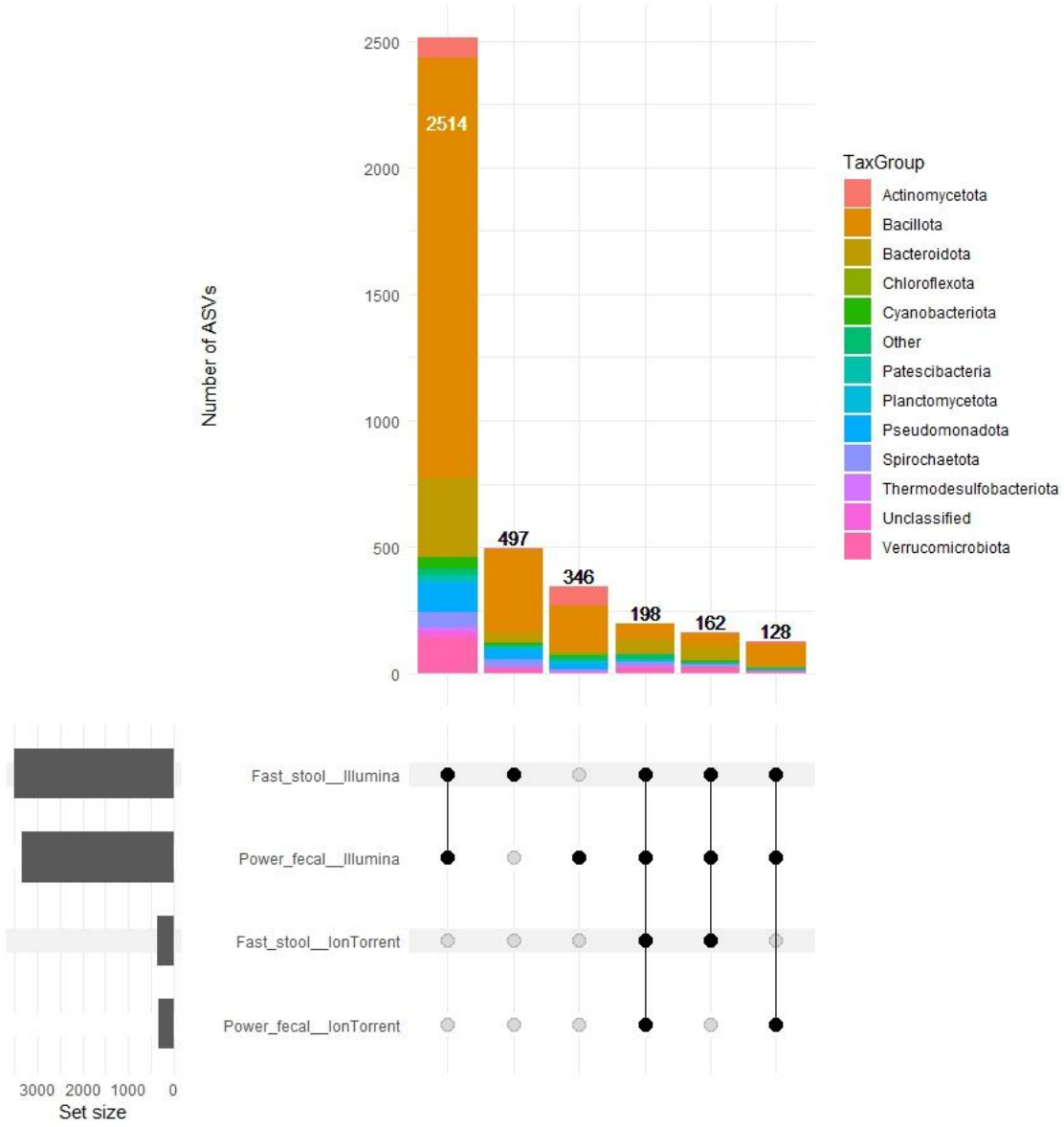



**Figure 2. UpSet plot of shared and unique OTUs across methodological combinations.** UpSet diagram showing OTU intersections across combinations of sequencing platform (Illumina, Ion Torrent) and extraction kit (Fast Stool, PowerFecal Pro). Horizontal bars show the total number of OTUs detected per combination; filled intersections show shared OTU subsets.

## Mock community and controls

The ZymoBIOMICS Microbial Community Standard was processed across kit × platform combinations to verify amplification and sequencing performance. The observed mock community profiles deviated from the expected composition (see Supplementary Figure S7). However, the overall pattern was highly consistent across sequencing platforms; differences between extraction kits were more pronounced for several families. Negative controls (extraction blanks) consistently yielded very low read counts and showed no recurrent OTUs at appreciable abundance; no further contaminant modeling was applied, and controls were excluded from downstream analyses.

## Gram status kit effect

Within the predefined interest set (n = 19 families), gram-positive taxa were more abundant with Power_Fecal, whereas gram-negative taxa tended to favor Fast_Stool, as shown in Figure 3. The median log2-fold change was greater for gram-negative bacteria than for gram-positive bacteria (Wilcoxon rank-sum p = 0.025), indicating a significant difference between Gram groups. Fisher's exact test of significant taxa confirmed a nonrandom association between Gram status and the kit effect (p = 0.031).

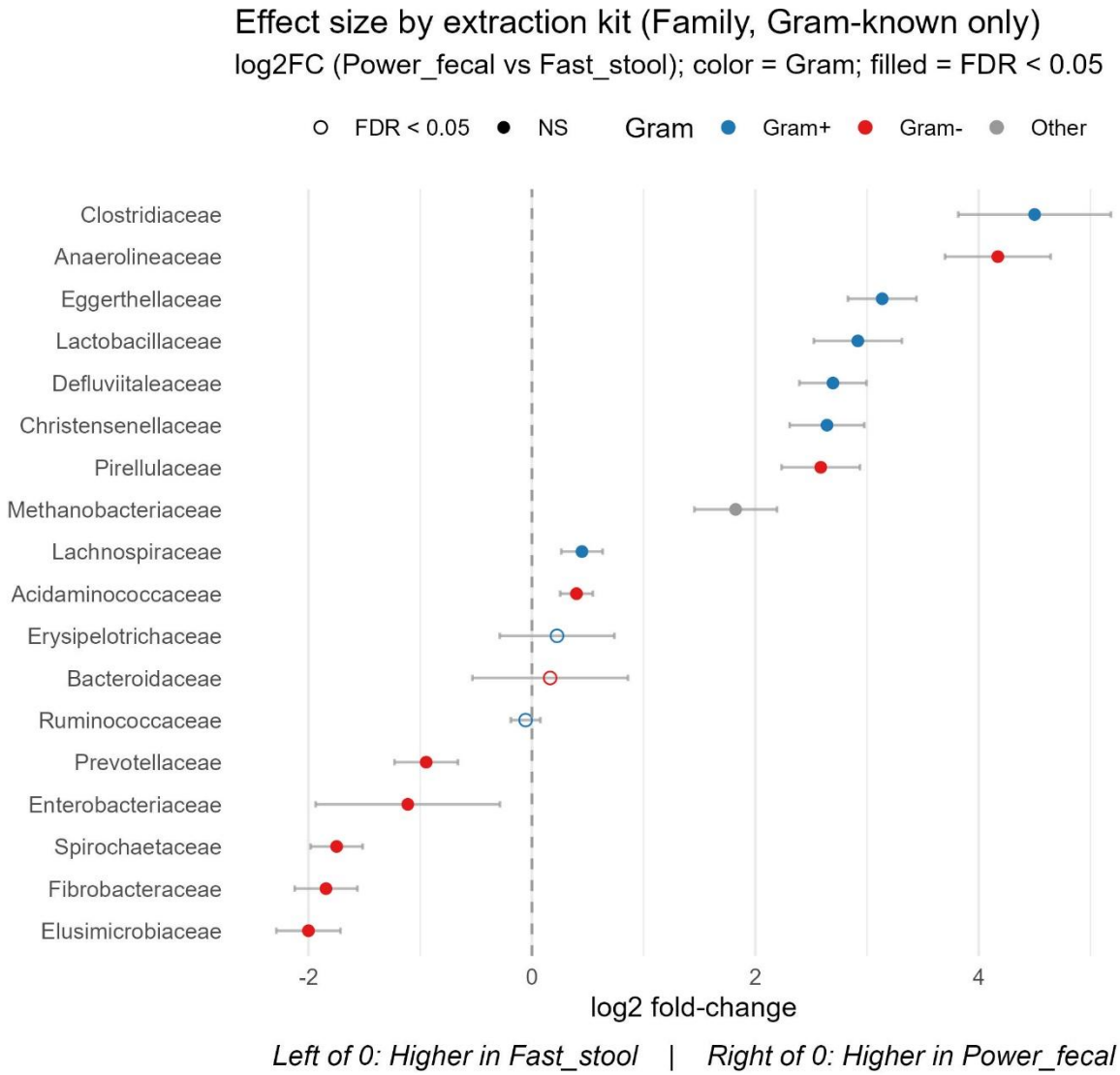



**Figure 3. Family-level extraction-kit effects.** Forest plot showing $\log_2$ fold-changes (PowerFecal Pro vs Fast Stool) for 19 predefined bacterial families. Points are colored by Gram status (blue: Gram-positive; red: Gram-negative). Filled points indicate families with FDR < 0.05. Horizontal lines represent 95% confidence intervals, and the vertical dashed line marks $\log_2$FC = 0. Positive values indicate higher abundance in Power_fecal, negative values indicate higher abundance in Fast_stool.

## Biological replicates across sample processing and platforms

The results of the ordination analyses revealed strong within-horse clustering across both sequencing platforms (Figure 4). Biological replicates (BR1–BR3) and extraction replicates (E1–E3) grouped closely within horses, and extraction kits did not disrupt this pattern. The first two PCoA axes explained 31.5% and 18.4% of the variation for Illumina and 19.1% and 13.1% for Ion Torrent. Global

PERMANOVA revealed similar total explained variation (Illumina 28.99%; Ion Torrent 31.07%; p = 0.001), with horse identity accounting for most of this variation (Illumina $R^2$ = 24.9%; Ion Torrent $R^2$ = 26.9%). No significant differences were detected among the biological or extraction replicates (p > 0.13 for Illumina; p > 0.48 for Ion Torrent).

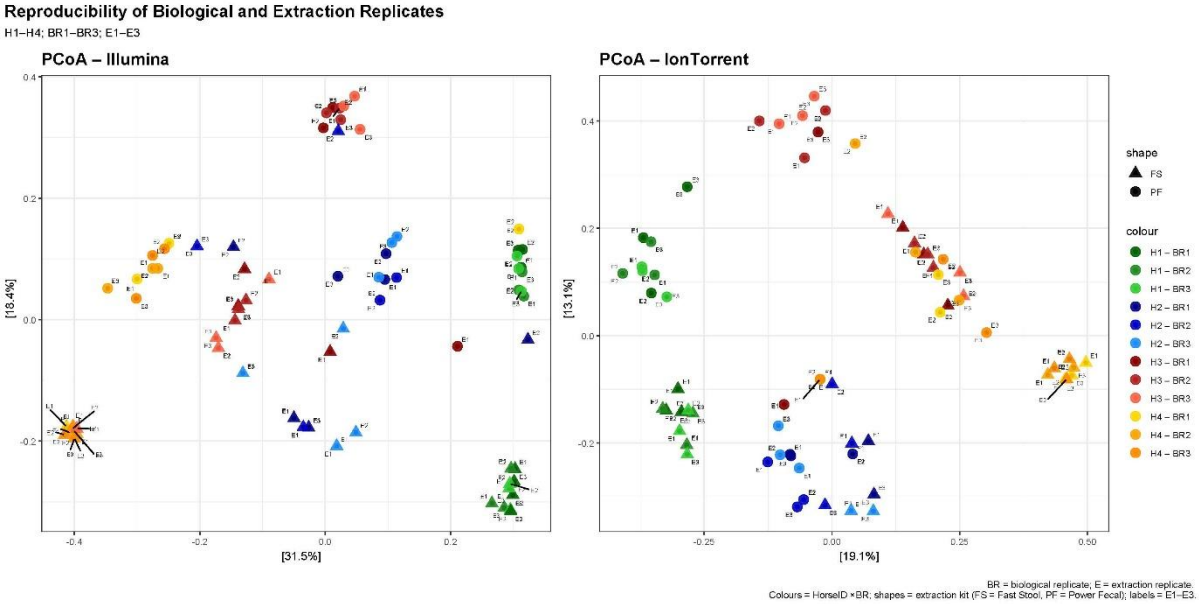



**Figure 4. Reproducibility of biological and extraction replicates.** PCoA of Bray–Curtis dissimilarities for four horses (H1–H4) sequenced on (A) Illumina and (B) Ion Torrent. Colors represent horse × biological replicate; shapes represent extraction kit (PF = PowerFecal Pro kit and FS = Fast stool kit); labels indicate extraction replicates (E1–E3). Ellipses show 68% normal confidence intervals.

## Impact of long-term storage in ethanol on microbiota profiles

Ethanol--preserved samples stored at 4 °C or room temperature (~21 °C) for up to 13 months presented modest shifts in community composition. The taxonomic composition across storage conditions is shown in Figure 5. PERMANOVA revealed significant effects of storage condition ($R^2$ = 0.083, $p$ < 0.0001) and extraction date ($R^2$ = 0.097, $p$ < 0.0001), while horse identity accounted for most residual variation. There were no differences in within-group dispersion across storage conditions ($p$ = 0.18) or extraction dates ($p$ = 0.11). The dispersion values per date and storage condition are shown in Supplementary Figure S8, and the PCoA ordinations for the same samples are shown in Supplementary Figure S9.

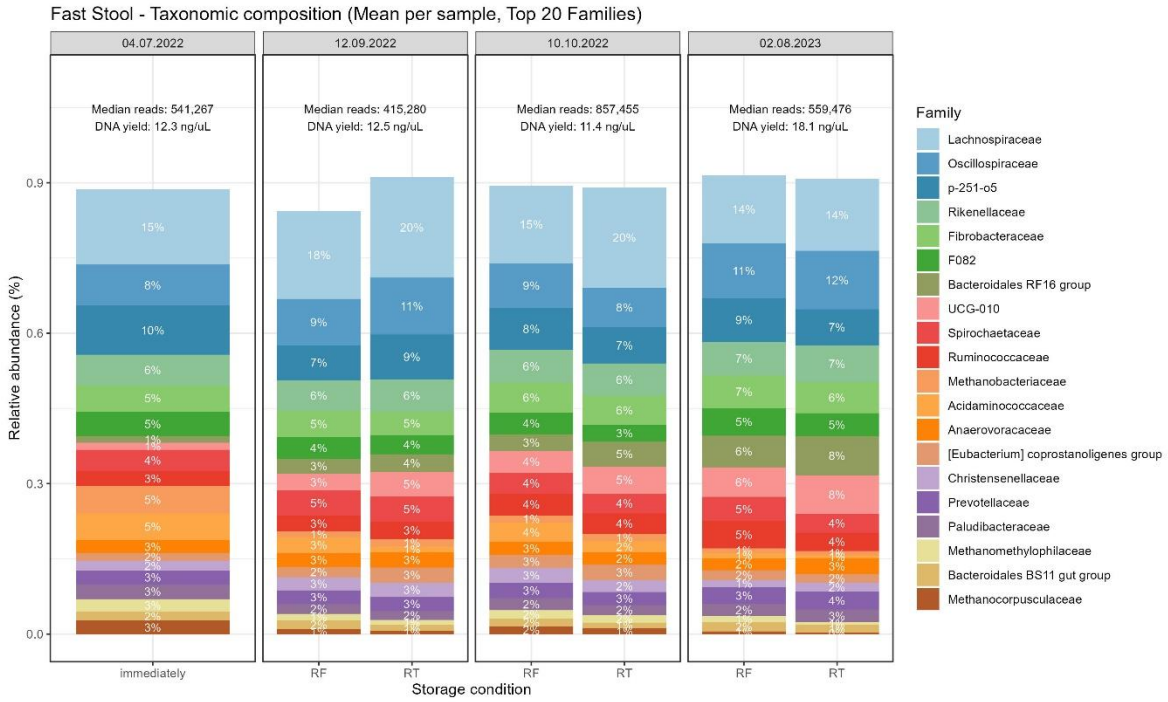



**Figure 5.** Taxonomic composition across storage conditions and extraction dates. Stacked barplots showing the mean relative abundance of the 20 most abundant bacterial families across baseline (04.07.2022) and stored samples (RT, RF) collected at later extraction dates (12.09.2022, 10.10.2022, 02.08.2023). Percent labels indicate proportional family contributions; median sequencing depth and mean DNA yield are displayed above panels.

### Sample collection

Pooled collection samples were compared to the mean profile of their biological replicates via Spearman correlation, Bray–Curtis dissimilarity, and presence/absence agreement (> 0.1%). The collected samples presented a median Spearman ρ = 0.72 (IQR 0.68–0.76), a Bray–Curtis score of 0.24 (IQR 0.23–0.25), and high taxonomic agreement (precision 0.92, recall 0.95).

## Discussion

### Dominance of horse identity

Across analyses, horse identity explained substantially more of the variation than extraction chemistry, platform and storage did, which is consistent with large multi-lab and cohort efforts showing that biological identity typically explains more variance than technical factors when workflows are applied consistently [17, 24, 25, 36]. The tight clustering of both the biological and extraction replicates indicates that each horse's profile is stable over the repeated-sampling interval and resilient to upstream steps. When an individual-level structure is pronounced, method-dependent noise is unlikely to override the biological signal, so cross-sectional comparisons are more likely to reflect host-associated structure rather than artifacts from extraction, platform or storage. Practically, these findings support longitudinal monitoring, dietary interventions and performance-oriented research where robust tracking of horse-specific profiles is essential. The ecological background supports this pattern: horses rely on a large and stable fermentation chamber dominated by fibrolytic and obligate anaerobic taxa, and as described by Costa and Weese (2012)

and Garber et al. (2020), such a system tends to promote stable host-associated communities [48, 52].

## Methodological factors shaping community profiles

### Sequencing platform

The platform differences were systematic but smaller than the host effects. Known contrasts in chemistry and error profiles explain why richness and dispersion differ between Illumina and Ion Torrent [44, 45]. Sequencing-depth differences likely explain why some phyla were detected only on Illumina, underscoring that platform choice becomes particularly important when studies aim to resolve low-abundance or hard-to-detect taxa. Despite these quantitative differences, platform choice did not disrupt the underlying biological signal: samples clustered by horse within both datasets, and mock communities showed similar deviation patterns on both platforms. This suggests that while platform-specific biases influence richness and dispersion, they do not obscure major ecological structure when laboratory workflows are applied consistently.

### DNA extraction kit

The extraction kits introduced consistent but heterogeneous shifts in community composition. Several gram-positive families were more abundant with PowerFecal Pro, whereas gram-negative families tended to be more abundant with Fast Stool extraction, which aligns with known lysis and inhibitor removal differences between kits [42, 43]. In line with these mechanisms, chemical and thermal lysis approaches can systematically overrepresent gram-negative groups and underrepresent gram-positive lineages, including those within Firmicutes and Actinobacteria [42, 68]. This taxon-specific sensitivity to lysis is particularly relevant for equine-associated fibrolytic families such as Lachnospiraceae and Ruminococcaceae, which may be underestimated when lysis is insufficient [8, 42, 43]. Consistent with these shifts, dispersion was heterogeneous within kits, indicating that the lysis strategy can affect not only central tendencies but also the spread of community profiles across samples. Because these shifts were consistent across platforms, extraction is an upstream driver of technical variation that can propagate into downstream analyses. This has been demonstrated in several interlaboratory benchmarking studies, where extraction kit choice contributed more to variation in microbiota profiles than the sequencing platform did [24, 36, 40, 42]. Moreover, the tight clustering of extraction replicates within horses shows that standardized workflows are internally reproducible once biological variation is accounted for [17, 36]. Taken together, the results highlight that the extraction method is one of the most influential methodological factors in fecal microbiota studies and should be standardized whenever possible.

### Storage in ethanol

Long-term ethanol storage produced modest compositional shifts that were small relative to the strong horse-specific structure and were not attributable to increased dispersion. These findings align with reports of short-term stability at 4 °C [69], although long-term ethanol effects remain less characterized, particularly in nonhuman hosts. Moossavi et al. (2019) reported similar trends, noting that ethanol performs comparably to freezing over short and moderate intervals, with minimal changes in community structure [70]. Our results extend these findings to equine feces, showing that even after thirteen months of storage, the horse-specific signal remained stronger than any storage-related effects. These findings suggest that ethanol preservation is a feasible approach for long-term equine fecal microbiota studies when immediate freezing is impossible, provided that laboratory workflows remain consistent and that storage metadata are incorporated into analytical models. However, the earliest post-sampling interval was not captured here, and rapid shifts immediately

after collection cannot be excluded. Nonetheless, the overall pattern indicates that long-term ethanol storage did not obscure the main biological structure of the dataset.

### Replicates and collection samples

The reproducibility of biological replicates supports the assumption of low within-event heterogeneity when sampling is performed consistently, as shown in other studies [17, 24]. The stability of pooled collection samples relative to their replicates mirrors the results from human microbiome research [71] and other species [72, 73]. Aguirre et al. (2014) reported that pooled fecal material can preserve major compositional patterns while reducing the sequencing burden, provided that the pooling is carefully controlled [71]. In our case, pooled samples effectively captured the main structure of the individual replicates, making them a reasonable proxy for studying storage-related effects. We note that this conclusion applies specifically to the Illumina workflow used here. Nevertheless, this is encouraging for studies relying on repeated sampling from the same individuals or using pooled collection samples to reduce sequencing effort.

## Methodological implications

Together, these findings highlight several practical recommendations for equine fecal microbiota studies.

I. Standardize extraction within studies, since kit-specific biases, especially those related to Gram status, were more pronounced than platform differences for several taxa.
II. Keep the sequencing platform consistent within studies, because platform choice shifts richness and dispersion even when the biological structure persists. Platform choice especially matters when studies target rare taxa, where differences in sequencing depth can influence detectability.
III. Include mock community controls for each extraction kit and each sequencing batch to reveal extraction specific deviations that platform comparisons alone may not capture.
IV. Use ethanol pragmatically for long term preservation when freezing is not feasible, but record storage duration and temperature and incorporate them into analytical models.

## Limitations and future directions

This study included four horses and pooled extract material for long-term storage assessment, limiting the ability to evaluate finer-scale variation across individuals and extraction replicates in stored samples. Storage tests were performed only on Illumina-sequenced samples and only with Fast Stool, and future work should expand platform–kit combinations for storage conditions. Additional studies could also examine the effects of bead-beating intensity or lysis chemistry on the recovery of specific taxa, especially Gram-positive groups. Larger datasets with dietary metadata and complementary shotgun metagenomics would allow deeper insight into functional and taxonomic stability across methodological choices.

# Conclusion

In equine fecal 16S profiling, individual horse identity dominates community structure, whereas platform, extraction kit and long-term ethanol storage introduce smaller, systematic shifts that can be managed by workflow standardization and appropriate modeling. This host-over-method hierarchy supports robust intra-individual tracking, reliable cross-sectional comparisons under consistent laboratory protocols, and pragmatic use of ethanol-preserved samples in field-oriented equine studies.

# Declarations

## Ethical statement and consent to participate

This study involved the non-invasive collection of freshly voided feces from privately owned horses. No animals were handled, restrained, or subjected to any procedures. According to Norwegian legislation (Forsøksdyrforskriften § 2 f), such non-invasive sampling does not constitute an animal experiment and therefore does not require ethical approval. This interpretation was confirmed in writing by the Norwegian Food Safety Authority (Mattilsynet) on 17 February 2022. All horse owners provided informed consent for the use of their animals' fecal samples in this study.

Because sampling occurred on private property with the owners' permission and did not involve any disturbance to the animals, no additional permits or field collection licenses were required. The study complied with all relevant institutional, national, and international guidelines for research involving animals.

## Consent for publication

Not applicable.

## Availability of data and materials

Raw sequencing data (FASTQ files generated on the Illumina NovaSeq 6000 and Ion S5 platforms) are publicly available in the European Nucleotide Archive (ENA) under project accession number PRJEB108720 (ERP189557). Ion Torrent runs are deposited under accessions ERR16777660–ERR16777661, and Illumina runs under ERR16777678–ERR16777687. Sample registration is available under accession ERS29282804 and the submission record under ERA35893055.
Minimal sample and sequence metadata adhering to MIxS/MIMARKS standards are archived in the USN Dataverse under DOI https://doi.org/10.18710/7HQACH.

## Competing interests

The authors declare that they have no competing interests.

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## Author Contribution

Sofie Geck Sevatdal (SGS): conceptualization; methodology; investigation; formal analysis (R); data curation; visualization; writing—original draft; writing—review & editing.
Jørn Henrik Sønstebø (JHS): conceptualization; methodology; investigation (laboratory); supervision
Anders Kristian Krabberød (AKK): formal analysis (R); supervision; writing—review & editing.
Mona Støren Sæbø (MSS): conceptualization; project administration; supervision; writing—review & editing.

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## Use of artificial intelligence

Microsoft Copilot (GPT-5) and ChatGPT (GPT-4) were used for language refinement, text restructuring, and technical assistance with R code (e.g., debugging and syntax suggestions). All scientific judgment, data analysis, and source selection were performed by the authors.

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# Main figure legends

**Figure 1. Variance explained by PERMANOVA factors.** Barplot showing marginal $R^2$ values for Model 1 (Illumina + Ion Torrent; permutations stratified by horse) and Model 2 (Illumina only; permutations stratified by library). Significance levels: ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05. PERMDISP results indicated dispersion differences for platform but not for storage.

**Figure 2. UpSet plot of shared and unique OTUs across methodological combinations.** UpSet diagram showing OTU intersections across combinations of sequencing platform (Illumina, Ion Torrent) and extraction kit (Fast Stool, PowerFecal Pro). Horizontal bars show the total number of OTUs detected per combination; filled intersections show shared OTU subsets.

**Figure 3. Family-level extraction-kit effects.** Forest plot showing $\log_2$ fold-changes (PowerFecal Pro vs Fast Stool) for 19 predefined bacterial families. Points are colored by Gram status (blue: Gram-positive; red: Gram-negative). Filled points indicate families with FDR < 0.05. Horizontal lines represent 95% confidence intervals, and the vertical dashed line marks $\log_2FC = 0$. Positive values indicate higher abundance in Power_fecal, negative values indicate higher abundance in Fast_stool.

**Figure 4. Reproducibility of biological and extraction replicates.** PCoA of Bray–Curtis dissimilarities for four horses (H1–H4) sequenced on (A) Illumina and (B) Ion Torrent. Colors represent horse × biological replicate; shapes represent extraction kit; labels indicate extraction replicates (E1–E3). Ellipses show 68% normal confidence intervals.

**Figure 5. Taxonomic composition across storage conditions and extraction dates.** Stacked barplots showing the mean relative abundance of the 20 most abundant bacterial families across baseline (04.07.2022) and stored samples (RT, RF) collected at later extraction dates (12.09.2022, 10.10.2022,

02.08.2023). Percent labels indicate proportional family contributions; median sequencing depth and mean DNA yield are displayed above panels.

# Supplementary figure legends

**Supplementary Figure S1. Observed OTU richness by sequencing platform.** Violin and boxplots showing observed OTU richness for Illumina and Ion Torrent libraries. Illumina consistently yielded higher OTU counts across samples.

**Supplementary Figure S2. Platform comparison by PCoA (non-rarefied and rarefied).**
Bray–Curtis PCoA of Library 1 samples sequenced on Illumina and Ion Torrent.
(A) Non-rarefied relative abundances.
(B) Rarefied to 10,000 reads per sample.
Points colored by platform; shapes correspond to horses (H1–H4).

**Supplementary Figure S3. Phylum-level differences between sequencing platforms.** Barplot showing the top 10 phylum-level differences in relative abundance (Illumina − Ion Torrent) across matched samples. Colors indicate direction of change (blue: Illumina > Ion Torrent; red: Ion Torrent > Illumina).
**Supplementary Figure S4.** PCoA of extraction-kit effects by horse. PCoA of Bray–Curtis dissimilarities, faceted by horse (H1–H4). Points are colored by extraction kit (Fast Stool, PowerFecal Pro). Ellipses represent 65% confidence intervals.

**Supplementary Figure S5. Extraction-kit comparison: alpha-diversity and DNA concentration**
(A) Alpha diversity (Shannon) for samples extracted with *Fast Stool* or *Power Fecal*. Each point represents one biological replicate, grouped by horse (H1–H4), with lines connecting samples from the same horse.
(B) DNA concentration (log10) measured prior to PCR in ng/µl, shown for the same samples. Colors indicate horse identity (H1–H4). Both panels show paired within-horse comparisons to illustrate extraction kit effects independent of host-specific variation.

**Supplementary Figure S6:** Volcano plot of differential abundance results from DESeq2 at the Family level for the contrast DNeasy PowerFecal Pro vs QIAamp Fast DNA Stool Mini. Each point represents a family; red indicates FDR < 0.05. Vertical dotted lines mark log2 fold-change = ±1 as a biologically relevant threshold. Top 10 significant families are annotated.

**Supplementary Figure S7. Mock community: expected vs observed composition.** Expected vs observed composition of mock community in positive controls, grouped by extraction kit and sequencing platform. Each panel represents a sequencing platform (Illumina, Ion Torrent) or the expected mock community composition. Bars show the relative abundance (%) of seven bacterial families included in the mock community. Colors indicate the source: grey for expected composition, blue for Fast Stool kit, and orange for Power Fecal kit.

**Supplementary Figure S8. Dispersion across storage conditions.** Boxplots showing within-group Bray–Curtis dispersion for samples processed at each extraction date, stratified by storage condition (immediate, RF, RT).

**Supplementary Figure S9. Storage-condition PCoA.** Bray–Curtis PCoA ordination of samples preserved in ethanol and extracted at multiple timepoints. Ellipses represent 68% confidence intervals for each storage group (immediate, RF, RT).

# Supplementary table legends

**Supplementary Table S1.** List of 19 bacterial families included in the predefined interest set used to evaluate extraction-kit effects.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1bacteriaofinterest.xlsx
- SupplementaryTablesInterestlistS4.docx
- SupplementaryMethodsS1S3.docx
- SupplementaryTablesInterestlistS4.pdf
- SupplementaryMethodsS1S3.pdf